Toll-Like Receptor 4 Is Involved in Outward Arterial Remodeling

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Background—Toll-like receptor 4 (Tlr4) is the receptor for exogenous lipopolysaccharides (LPS). Expression of endogenous Tlr4 ligands, heat shock protein 60 (Hsp60) and extra domain A of fibronectin (EDA), has been observed in arthritic and oncological specimens in which matrix turnover is an important feature. In atherosclerosis, outward remodeling is characterized by matrix turnover and a structural change in arterial circumference and is associated with a vulnerable plaque phenotype. Since Tlr4 ligands are expressed during matrix turnover, we hypothesized that Tlr4 is involved in arterial remodeling.

Methods and Results—In a femoral artery cuff model in the atherosclerotic ApoE3 (Leiden) transgenic mouse, Tlr4 activation by LPS stimulated plaque formation and subsequent outward arterial remodeling. With the use of the same model in wild-type mice, neointima formation and outward remodeling occurred. In Tlr4-deficient mice, however, no outward arterial remodeling was observed independent of neointima formation. Carotid artery ligation in wild-type mice resulted in outward remodeling without neointima formation in the contralateral artery. This was associated with an increase in Tlr4 expression and EDA and Hsp60 mRNA levels. In contrast, outward remodeling was not observed after carotid ligation in Tlr4-deficient mice.

Conclusions—These findings provide genetic evidence that Tlr4 is involved in outward arterial remodeling, probably through upregulation of Tlr4 and Tlr4 ligands. (Circulation. 2004;109:393-398.)

Key Words: remodeling ■ arteries ■ plaque ■ atherosclerosis ■ proteins

Toll-like receptor 4 (Tlr4) is the receptor for exogenous lipopolysaccharide (LPS), endogenous heat shock protein 60 (Hsp60), and extra domain A of fibronectin (EDA). Tlr4 expression has recently been described in atherosclerotic arteries in endothelial cells, macrophages, and adventitial fibroblasts. Using a mouse femoral cuff model, we recently demonstrated that Tlr4 is involved in neointima formation. Moreover, Tlr4 polymorphism is associated with carotid intima thickness in humans. However, a function for Tlr4 in arterial geometrical remodeling, the other determinant for arterial lumen loss, is unknown.

Expression of endogenous Tlr4 ligands, Hsp60 and EDA, has been observed in arthritic and oncological specimens in which matrix turnover is an important feature.

Matrix turnover in arteries occurs during arterial remodeling in response to sustained blood flow changes, balloon injury, and atherosclerotic plaque formation. Arterial remodeling, which can be outward to reduce lumen loss and inward to increase lumen loss, is characterized by structural changes of the arterial wall involving cell migration and collagen (matrix) breakdown by matrix metalloproteases (MMPs), including MMP9, which can be stimulated by Tlr4 activation.

Outward remodeling in arteries is an important determinant for lumen loss because it can compensate for plaque accumulation in the arterial lumen. However, although the luminal area is preserved, the plaque beneath the surface of the lumen often has a vulnerable plaque phenotype. Next to a vulnerable plaque phenotype, outward remodeling is associated with aneurysm formation and shear stress–induced arteriogenesis.

Previous observations revealed enhanced expression of Tlr4 ligands in non–vascular tissue remodeling. In the current study, we hypothesized that Tlr4 is involved in arterial...
remodeling. We studied arterial plaque formation and subsequent arterial remodeling after Tlr4 activation in the atherosclerotic ApoE3 Leiden mouse. We investigated the involvement of Tlr4 in femoral artery remodeling with neointima formation and carotid artery remodeling without neointima formation by using wild-type and Tlr4-deficient mice. Furthermore, expression and localization of carotid Tlr4 was determined and Hsp60 and EDA mRNA levels were measured. This revealed that Tlr4 is involved in the outward arterial remodeling process and shows that Tlr4 and its endogenous ligands are upregulated during remodeling.

Methods

Animal Experiments
Femoral cuff placement was performed in male ApoE3 Leiden mice that were crossed for 18 generations with C57BL/6 mice (8 to 12 weeks old) and received a high-fat, cholesterol-rich (HFC) diet throughout the experimental period starting 4 weeks before surgery, as described before.18 LPS (1 μg/μL) or PBS (control) dissolved in gelatin was administered inside the cuff; mice were killed 3 weeks after cuff placement (+LPS, n=10; control mice, n=10).

Femoral cuff placement with or without LPS (1 μg/μL) was also performed in female BALB/c (n=18) and C.C3H-Tlr4<sup>−/−</sup> mice (Tlr4-deficient mouse on a Balb/c background, Jackson Laboratory, n=18; age, 12 to 20 weeks) and killed 3 weeks after cuff placement.<sup>6</sup>

Carotid artery ligation was performed as described by de Kleijn et al<sup>19</sup> in 14 BALB/c and 17 C.C3H-Tlr4<sup>−/−</sup> mice by using the carotid artery contralateral to the ligation as a model for outward remodeling. Nonligated female BALB/c and C.C3H-Tlr4<sup>−/−</sup> mice served as control (BALB/c n=5; C.C3H-Tlr4<sup>−/−</sup> n=5). All arteries used for morphometry were perfusion-fixed first for 3 minutes through the left ventricle with PBS plus 10 to 4 mol/L nitroprusside at 99 mL/h to get maximal dilation of the arteries and normal intraventricular pressure for nitroprusside-treated mice (50 to 60 mm Hg<sup>24</sup>). Intraventricular pressure was measured with a pressure sensor with data recorder (Spacelabs) connected between the pressure sensor and the needle in the left ventricle. This was followed by a 3-minute perfusion with 4% paraformaldehyde in PBS plus 10 to 4 mol/L nitroprusside at 99 mL/h to fix the arteries at maximal dilation and normal pressure.

Carotid ligation was also performed in another group of 60 female BALB/c mice to measure carotid RNA and protein levels at 0, 3, 5, 8, 20, and 28 days after ligation (n=10/time point), as described previously by de Kleijn et al.<sup>21</sup>

The ethics committee on animal welfare of TNO (The organization for Applied Scientific research) and the Utrecht University approved the animal experiments.

Definition of Arterial Remodeling
Arterial remodeling is defined as a change in arterial size compared with a control or reference artery. An increase in size is defined as outward remodeling, whereas a decrease in size is defined as inward remodeling. As a measure of arterial size, we used the external elastic lamina (EEL) area calculated from the EEL length including corrugations. For the cuff models, the EEL areas are compared between the cuff with and without LPS (reference). For the ligation model, EEL areas are compared between the carotid artery contralateral to the ligated carotid artery and unligated carotid arteries (reference).

Morphological Quantification in Sections of Cuffed Femoral Artery and Contralateral Unligated Carotid Artery
Paraffin sections were stained with either elastin–van Gieson or HPS. Ten equally spaced (200 μm) cross sections were used in ApoE3 Leiden mice and 3 to 6 in the BALB/c and C.C3H-Tlr4<sup>−/−</sup> mice to quantify intimal lesions and EEL. Using image analysis software (Leica or Analytis), total cross-sectional medial area was measured between the external and internal elastic lamina; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

Quantitative RT-PCR
Tripuri (Roche) was used for isolation of RNA and protein according to the manufacturer’s protocol. Mouse Tlr4 (forward: 5'-tacctgctgctcatac-3'; reverse: 5'-caagatataccaagcggct-3') EDA (forward: 5'-actgtggttagtgatgctc-3'; reverse: 5'-tgaagtcactacactcag-3'), Hsp60 (forward: 5'-aagctatggtaagcagggag-3'; reverse: 5'-cagcaatattacaaactg-3'), and 18S (forward: 5'-tcaacaggga-aaactcc-3'; reverse: 5'-accagacaagtcctccac-3') primers were designed with the use of the Primer Program at CMBI (Nijmegen). cDNA synthesis, and quantitative PCR was performed as described before.<sup>21</sup>

Western Blotting
Denatured samples (6 μg/lane) with β-mercaptoethanol were separated on a 10% SDS polyacrylamide gel and blotted to a Hybond-C membrane (Amersham Pharmaceuticals). Blocking and incubation steps were done in 5% defatted dry milk in PBS/0.1% Tween 20. Blots were incubated with rabbit anti-human Tlr4 (1:500, Santa Cruz Biotechnology), biotin-labeled swine anti-rabbit (1:2000, DAKO) followed by streptavidin horseradish peroxidase (1:1000, Vector Laboratory). Bands were visualized with the use of the ECL kit (Amersham). Optical density of Tlr4-positive bands was measured with the use of the GelDoc system (Biorad) and expressed in arbitrary units.

Immunohistochemistry
Mouse femoral or carotid arteries were embedded in paraffin. Four-micrometer sections were cut and deparaffinized. Endogenous peroxidase was quenched in MeOH containing 1.5% H<sub>2</sub>O<sub>2</sub> for 30 minutes at room temperature. Antigen retrieval was performed by boiling sections for 20 minutes in 10 mmol/L citrate buffer, pH 6.0. Sections were blocked with 10% normal swine serum and incubated overnight at 4°C with rabbit anti-human Tlr4 immune serum (1:50, Santa Cruz Biotechnology, Inc). Sections were then incubated 1 hour at room temperature with biotin labeled swine anti-rabbit (1:1000, DAKO) followed by streptavidin horseradish peroxidase (1:1000, Vector Laboratory). As a negative control, the immune serum was replaced with nonimmune rabbit IgG (4 μg/mL, Vector Laboratory). Tlr4 was visualized with AEC staining (red), and nuclear staining was performed with hematoxylin.

Statistics
All data are presented as mean±SEM. The Mann-Whitney test was used for all experiments except for the mRNA and protein measurements. To determine whether there was an upregulation in mRNA or protein level over time, the logarithm was taken and ANOVA with Bonferroni post hoc test was performed. A value of P<0.05 was regarded as significant.

Results

Tlr4 Stimulation in an Atherosclerotic Mouse Model
After 3 weeks, stimulation with LPS of the cuffed femoral artery in ApoE3 Leiden mice on a HFC diet resulted in a larger EEL area compared with the group without LPS application (Table, P=0.009, Figure 1). The group with LPS application also showed a larger plaque area, mainly consisting of accumulated smooth muscle cells and foam cells as described before,<sup>21</sup> compared with the group without LPS application (Table, P=0.011, Figure 1). The cholesterol levels are comparable in both groups (control group, 17.7±7.9 mmol/L versus LPS group, 16.3±4.6 mmol/L).
Tlr4 and Outward Arterial Remodeling in a Femoral Cuff Model With Neointima Formation

The femoral cuff model in BALB/c and C.C3H-Tlr4 LPS-d mice (a Tlr4-deficient mouse on a BALB/c background) showed that after LPS stimulation, wild-type mice revealed a larger EEL area in comparison to the nonstimulated wild-type mice (Table, $P=0.019$, Figure 2A). The C.C3H-Tlr4 LPS-d mice, however, did not show an increased EEL after LPS stimulation compared with the nonstimulated C.C3H-Tlr4 LPS-d mice (Table and Figure 2A). Neointima formation was less in the Tlr4-deficient mice compared with the wild-type mice but is increased in the LPS-stimulated Tlr4-deficient mice compared with the nonstimulated Tlr4-deficient mice (Figure 2B).

The wild-type mice showed a correlation between the EEL area and intima area ($r=0.525$, $P=0.024$, Figure 2C), which was absent in the C.C3H-Tlr4 LPS-d mice (Figure 2D). These data suggest that Tlr4 is involved in the outward remodeling process in a model with neointima formation.

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### Table 1

<table>
<thead>
<tr>
<th></th>
<th>EEL</th>
<th>Media</th>
<th>Intima/Plaque</th>
<th>Lumen</th>
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<tr>
<td><strong>Femoral cuff</strong></td>
<td></td>
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<tr>
<td>ApoE3 (gel)</td>
<td>28.226±2.217</td>
<td>11.660±0.700</td>
<td>8.448±1.902</td>
<td>8.118±1.149</td>
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<td>ApoE3 (LPS)</td>
<td>38.784±2.415</td>
<td>14.131±0.897</td>
<td>15.803±1.565</td>
<td>8.770±1.137</td>
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<tr>
<td>Wild-type (gel)</td>
<td>22.789±3.076</td>
<td>8.985±0.845</td>
<td>2.353±1.076</td>
<td>1.149±0.265</td>
</tr>
<tr>
<td>Wild-type (LPS)</td>
<td>30.956±2.485</td>
<td>9.624±0.387</td>
<td>9.133±1.714</td>
<td>12.198±1.595</td>
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<tr>
<td>Tlr4-deficient (gel)</td>
<td>16.241±1.262</td>
<td>7.976±0.334</td>
<td>1.279±0.183</td>
<td>6.986±0.197</td>
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<tr>
<td>Tlr4-deficient, (LPS)</td>
<td>17.213±2.275</td>
<td>8.001±0.420</td>
<td>3.859±0.912</td>
<td>5.354±0.154</td>
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|                |         |         |               |         |
| **Carotid ligation** |         |         |               |         |
| Wild-type, unligated | 11.783±0.1577 | 18.039±2.377 | 0.086±0.0112 | 9.799±1.7634 |
| Wild-type, ligated   | 14.169±0.5644 | 24.735±1.279 | 0.423±0.4833 | 11.433±0.4833 |
| Tlr4-deficient, unligated | 13.373±1.3300 | 21.764±1.881 | 0.090±0.0112 | 11.409±1.1638 |
| Tlr4-deficient, ligated | 14.125±0.5509 | 23.770±1.086 | 0.019±0.0112 | 11.355±0.4915 |

Data are mean±SEM (in μm²), n=7 to 17. Outward remodeling of the carotid artery is determined in the carotid artery contralateral to the ligated artery.

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Figure 1. HPS staining of femoral artery cross sections of APOE3 Leiden mouse treated with periadventitial cuff and gelatin (A) and with cuff and gelatin containing LPS (B). Arrows indicate EEL. EEL area (C) and plaque area (D) of the femoral artery of APOE3 Leiden mice after cuff placement with or without stimulation of LPS. n=10 mice per group. *$P=0.009$, **$P=0.011$. Bar=50 μm.

Figure 2. EEL area (A) and intima area (B) of wild-type (Wt) BALB/c (black bar) and C.C3H-Tlr4 LPS-d (white bar) mice treated with periadventitial cuff containing gelatin with or without LPS.6 n=7 to 9 mice per group, *$P=0.019$. Intima area versus EEL area plot in wild-type mice (C) ($P=0.024$) and in C.C3H-Tlr4 LPS-d mice (D).
Expression of Tlr4 and Endogenous Tlr4 Ligands in a Flow Cessation Model

Next, we studied whether outward remodeling resulted in an increase of Tlr4 or endogenous Tlr4 ligand levels in the contralateral unligated carotid artery. We observed an upregulation of Tlr4 mRNA (∼25 times) and Tlr4 protein levels (∼3 times) over time that was significant at 28 days after ligation (P=0.046, P=0.029, respectively, Figure 4A). We also measured the EDA and Hsp60 mRNA levels at 0 and 28 days after ligation in wild-type BALB/c mice. Both endogenous Tlr4 ligands were significantly upregulated at 28 days (EDA, 4 times, P=0.007, and Hsp60, 24 times, P=0.041, Figure 4B).

Staining for Tlr4 protein expression demonstrated that the endothelial cell layer, media, and adventitia contained Tlr4-positive cells (Figure 4C, left panel). Negative control with nonimmune rabbit IgG showed no positive staining (Figure 4C, right panel) or bands after Western blotting (Figure 4A, top panel).

Discussion

Inappropriate arterial remodeling is currently thought to be the main cause of prevalent vascular pathologies, including atherosclerosis and restenosis, but the components and regulatory mechanisms of this process are still unclear.

First, we demonstrated that Tlr4 activation in an atherosclerotic (ApoE3 Leiden) mouse model can induce outward remodeling and plaque formation.

The increase in atherosclerotic plaque formation after LPS application is consistent with the formation of a smooth muscle cell–rich lesion after adventitial LPS application in rats and with the role of Tlr4 in neointima formation, as described before in mouse and plaque formation in humans.

Next, we questioned if Tlr4 is involved in outward arterial remodeling with neointima formation and used the femoral cuff model in BALB/c and C.C3H-Tlr4<sup>PS</sup>-d mice. This showed that Tlr4 is involved in outward remodeling. Although no relation was found between intima formation and outward remodeling in the C.C3H-Tlr4<sup>PS</sup>-d mouse, it remains difficult to determine what the contribution of neointima formation is in this process. For this, we investigated if Tlr4 is involved in outward arterial remodeling without neointima formation or injury. Thus, outward remodeling by lumen area increase depends on Tlr4 presence independent of neointima formation.

The finding that Tlr4 is involved in outward remodeling with and without neointima formation suggest an important role for Tlr4 in all outward arterial remodeling.

Previously, Kiechl et al demonstrated a relation between Asp299Gly Tlr4 polymorphism and carotid intima media thickness. It remains to be determined if human Asp299Gly Tlr4 polymorphism is also associated with less outward remodeling. Less outward remodeling might lead to more lumen loss, but, since outward remodeling is associated with an unstable plaque phenotype, this might lead to more stable plaques. The plaque phenotype is determined by collagen turnover and inflammation. Earlier findings show that Tlr4 is associated with collagen turnover, initiation of an inflammatory response, and production of chemokines and cytokines such as monocyte chemoattractant protein-1, suggesting a potential role for Tlr4 in plaque stabilization. Recently, Boekholt et al found that Tlr4 polymorphism modify the efficacy of statin therapy and the risk of cardiovascular events. This points again to a role of Tlr4 in cardiovascular disease, although the exact mechanistic role of Tlr4 remains obscure.

Since in the carotid ligation model no neointima formation occurs and no exogenous Tlr4 ligand was used, we studied whether outward remodeling resulted in an increase of Tlr4 or endogenous Tlr4 ligands levels. Tlr4 was upregulated on mRNA and protein levels that localized to the endothelial, medial, and adventitial layers. Also, two endogenous Tlr4 ligands, EDA and Hsp60, showed an increased mRNA expression at 28 days after ligation. This indicates that during outward arterial remodeling, endogenous ligands are the main Tlr4 activators, and no exogenous ligands are necessary, as was found earlier for murine atherosclerosis. Nevertheless, a causal role for endogenous Tlr4 ligands in remodeling still has to be established.
The involvement of Tlr4 in arterial remodeling might point to a role for Tlr4 in arteriogenesis in which outward remodeling is an essential process. Furthermore, Tlr4 activation results in increased production of monocyte chemoattractant protein-1 and other cytokines, which stimulates arteriogenesis. Similarly, a bolus of LPS improves peripheral and collateral conductance after femoral artery occlusion in the rabbit.
Limitations

Outward remodeling in the Tlr4-deficient mouse is not prevented by the femoral cuff, since in both ApoE3 Leiden (Figure 1C) and wild-type Balb/C (Figure 2a), outward remodeling occurs. However, we cannot exclude that the cuff prevents even more outward remodeling in ApoE3 Leiden and Balb/c.

Arteries were pressure-fixed with constant intraventricular pressure (≈50 mm Hg). Although we did not determine intraluminal arterial pressure, procedure and intraventricular pressure was the same in the different mice groups.

In summary, we showed that Tlr4 is involved in the outward arterial remodeling process, probably through up-regulation of Tlr4 and its endogenous ligands, EDA and Hsp60, which might also apply to arteriogenesis.

Acknowledgments

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References
